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Research Article

Negative impact of high doses of follicle-stimulating hormone during superovulation on the ovulatory follicle function in small ovarian reserve dairy heifers†

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Abstract

When women with small ovarian reserves are subjected to assisted reproductive technologies, high doses of gonadotropins are linked to high oocyte and embryo wastage and low live birth rates. We hypothesized that excessive follicle-stimulating hormone (FSH) doses during superovulation are detrimental to ovulatory follicle function in individuals with a small ovarian reserve. To test this hypothesis, heifers with small ovarian reserves were injected twice daily for 4 days, beginning on Day 1 of the estrous cycle with 35, 70, 140, or 210 IU doses of Folltropin-V (FSH). Each heifer ($n = 8$) was superovulated using a Williams Latin Square Design. During each superovulation regimen, three prostaglandin $F_{2\alpha}$ injections were given at 12-h interval, starting at the seventh FSH injection to regress the newly formed corpus luteum (CL). Human chorionic gonadotropin was injected 12 h after the last (8th) FSH injection to induce ovulation. Daily ultrasonography and blood sampling were used to determine the number and size of follicles and corpora lutea, uterine thickness, and circulating concentrations of estradiol, progesterone, and anti-Müllerian hormone (AMH). The highest doses of FSH did not increase AMH, progesterone, number of ovulatory-size follicles, uterine thickness, or number of CL. However, estradiol production and ovulation rate were lower for heifers given high FSH doses compared to lower doses, indicating detrimental effects on ovulatory follicle function.

Summary sentence

High doses of Folltropin-V in cattle with a low antral follicle count and small ovarian reserve are detrimental to ovulatory follicle function.

Key words: superovulation, estradiol, anti-Müllerian hormone, ovulation rate, ovulatory follicle function, number of ovulatory follicles, bovine.

Introduction

Assisted reproductive technologies (ARTs) include intravaginal ultrasound-guided needle aspiration of ovarian follicles to recover oocytes, also referred to as oocyte pick-up in cattle, or oocyte retrieval in women, for in vitro fertilization. ART also includes traditional embryo transfer primarily used in cattle to propagate offspring from genetically superior donors at a greater than normal rate [1]. Both ART techniques use exogenous follicle-stimulating hormone (FSH) to stimulate the growth of large numbers of ovulatory follicles to obtain oocytes for fertilization [2]. Response to superovulation, however, is highly variable among women [3] and cattle [2, 4], often resulting in costly, unpredictable outcomes [5–7]. Many reasons may exist for this variability, such as age [8, 9] and inherent differences in size of the ovarian reserve [10]. Several studies indicate that high FSH doses may contribute to the variability in superovulation response and outcomes. For example, dose–response studies in cattle indicate that high doses of FSH or FSH-like factors (e.g., pregnant mare serum gonadotropin, human menopausal gonadotropin) used to induce superovulation decrease fertilization rate [11–13], embryo yield [13, 14] or quality [11, 13], and number of transferable embryos [12–15]. High FSH doses are also linked to high oocyte and embryo wastage in cattle [11–17] and women [3, 5, 6, 18–22]. A recent study, with over 500 000 women, reports a negative relationship between FSH dose and live birth rate independent of age, weight, or health of the donor in women [3]. These data support the hypothesis that high FSH doses used to induce superovulation may be detrimental to ovulatory follicle function, which in turn may impair oocyte quality and embryo survival.

The large study supporting detrimental effects of high FSH doses on live birth rates during ART in women is correlative [3]. However, determination of the potential direct negative effects of excessive FSH doses on embryo survival in women is unlikely to occur in ART clinics. Consequently, we chose the bovine as an experimental model to examine FSH action during ovarian stimulation because the bovine is a single-ovulating species with a relatively long reproductive cycle like women; and during embryo transfer, the bovine undergoes ovarian stimulation (superovulation) protocols similar to those used during ART in women. In addition, a major contributing factor compelling women to seek ART is a small ovarian reserve [23]. Thus, another significant advantage of the bovine model is that we have established procedures to identify cattle with small ovarian reserves to evaluate FSH action during superovulation [24–27].

Variable results have emerged using the bovine model with respect to FSH dose on ART outcomes. Some studies reported no effect of high FSH doses on fertilization rate [28–31], embryo yield [28–32], or number of transferable embryos [11, 28–30, 32], but other studies reported negative effects of high FSH doses on embryo transfer outcomes [11–15]. Interestingly, some studies reported negative effects of high FSH doses on the number of corpora lutea (CL) [16, 29], circulating progesterone [16] and estradiol [29] concentrations, and ovulation rate [16]. Still other studies reported that high FSH doses increased the number of antral follicles [28, 30], estradiol [16], and progesterone concentrations [29], while others reported no effects of high FSH doses on CL number [11–13, 28, 30, 32], circulating progesterone [28] or estradiol [11] concentrations, or ovulation rate [11, 30]. Such variability among studies may reflect the use of diverse sources and doses of FSH or FSH-like products to superovulate cattle of various breeds, ages, or parities and with unknown numbers of follicles in the ovarian reserve. Moreover, the FSH dose–response studies typically used cross-sectional experimental designs with individual cattle assigned at random to one of several FSH doses with limited numbers of animals per dose (usually 4 or 5), single point in time measurements of most endpoints, different superovulation regimens starting at unknown stages of a follicular wave or CL development, and on different days of the estrous cycle.

To minimize the sources of variation and achieve a direct test of effects of high FSH doses on ovarian function in cattle, we employed a powerful experimental design that included Holstein heifers with small ovarian reserves and the use of a Williams Latin Square Design for statistical analyses. The small ovarian reserve model minimizes variability in the response to superovulation attributable to age-related differences in the ovarian reserve and mimics several characteristics of women with small ovarian reserves including a low antral follicle count (AFC, [24]), hypersecretion of FSH [24–26], low circulating anti-Müllerian hormone (AMH) concentrations [27] during the reproductive cycle, and poor response to superovulation [24]. To test our hypothesis, the same eight 11- to 12-month-old Holstein heifers were subjected to superovulation, beginning on Day 1 of the estrous cycle (near beginning of the first follicular wave) with four different FSH doses using a Williams Latin Square Design [33]. The Williams Latin Square Design was chosen because each animal serves as its own control, thus reducing the total number of animals needed to complete the dose–response study. Furthermore, because the same heifers were superovulated multiple times, the design balances and tests for potential carryover effects of one superovulation on response to the next superovulation. The objective of the present study was to determine the effects of different FSH doses used to superovulate cattle on ovulatory follicle development and function. This rigorous study design revealed significant negative effects of high FSH doses on ovarian function.

Materials and methods

Identification of heifers with small ovarian reserves

Our previous studies have established that 11- to 12-month-old heifers with ≤15 follicles of ≥3 mm in diameter during ovarian follicular waves (15–20% of a herd) have an 80% smaller ovarian reserve compared with age-matched counterparts with a high AFC (≥25 follicles) [24, 26, 27, 34–36]. To identify heifers with small ovarian reserves for the study, 79 Holstein heifers (11–12 months of age, weighing 318–408 kg), located at Green Meadow Farms Inc., Ovid-Elsie, MI, were subjected to ovarian ultrasonography to determine AFC and follicle sizes [26]. Approximately half of the heifers had an AFC of ≤10 follicles. These low AFC heifers received two 2 mL intramuscular injections of prostaglandin $F_{2\alpha}$ (PG, 12.5 mg PG/mL, Lutalyse *HighCon*, Zoetis) 10 days apart to induce luteolysis, which triggers ovulation and development of the first follicular wave of the estrous cycle. Four days after the last PG injection (about Days 1–2 of the estrous cycle and beginning of the first follicular wave), the heifers were subjected to ultrasonography to determine AFC. The result was that eight heifers were identified with an AFC ranging from 5 to 9 and subsequently housed at the Michigan State University Beef Cattle Teaching and Research Center for the duration of the project. These heifers were fed maintenance diets that met NRC requirements [37]. The Institutional Animal Care and Use Committee at Michigan State University sanctioned all procedures involving cattle.

Superovulation of heifers with small ovarian reserves

The heifers received three PG injections to synchronize estrous cycles. The first two injections were 10 days apart and the third PG injection

was given 12 h after the second PG injection. Each heifer was subjected to daily ultrasonography, beginning 36 h after the last PG injection to detect ovulation and the emergence of the first follicular wave. The heifers were then treated with Folltropin-V (Vetoquinol USA Inc.), an extract of porcine pituitary glands that contains 700 IU (equivalent to 400 mg NIH-FSH-P1 with 0.25% LH contamination or <1 mg NIH-LH-S19) of FSH per 20 mL vial. Hereafter, because of the minimal amount of LH contamination, Folltropin-V is referred to as FSH.

To minimize confounding variables and improve our understanding of the FSH dose effects on ovulatory follicle function, we selected a Williams Latin Square Design. Each of the eight heifers were superovulated a total of four times, but a different FSH dose was administered during the first, second, third, and fourth superovulation regimen. Furthermore, the sequence of FSH doses used to induce each superovulation differed for every animal. For example, as seen in Supplemental Table S1, the actual FSH dose sequence for Heifer #1 in our study was twice-daily injections for 4 days of 35, 70, 140, and 210 IU FSH used to induce their first, second, third, and fourth superovulation, respectively. In contrast, the actual FSH dose sequence for Heifer #2 in our study was twice-daily injections for 4 days of 140, 210, 35, and 70 IU FSH for the first, second, third, and fourth superovulations, respectively. It is to be noted that each dosage follows every other dosage treatment exactly twice, thereby being balanced for carryover effects. For example, the dosage of 70 IU follows the dosage of 35 IU twice, that is, once in Heifer #1 and again in Heifer #8 (Supplemental Table S1). Another advantage of this Latin Square Design is that it minimizes the potential impact of other nuisance variables, including differences in seasonal temperature, rations or animal genotypes, thereby facilitating greater precision on estimating the effects of the different FSH doses on the endpoints measured in our study [38]. Each heifer was injected intramuscularly twice daily (12 h apart) for 4 days (8 injections total) with one of the following four different FSH doses per injection: (1) 35 IU (20 mg NIH-FSH-P1/ml), (2) 70 IU (40 mg/2 mL), (3) 140 IU (80 mg/4 mL), and (4) 210 IU (120 mg/6 mL). To test our hypothesis and to avoid potential confounding effects of follicular responsiveness to decreasing FSH doses during superovulation, we chose to use eight equal dose injections of FSH instead of the decreasing dose regimen commonly used commercially during embryo transfer in cattle. Hereafter, the dose of FSH is referred to as IU per injection. The FSH dose range per injection was 60% lower and 240% higher than the Vetoquinol recommended dose per injection of 87.5 IU.

FSH injections began 36 h after the last PG injection which was ±1 day from ovulation and initiation of the first follicular wave in all heifers in our study. To regress the newly formed CL, a further three PG injections were given, 12 h apart starting at the time of the seventh FSH injection (about Days 4–5 of the estrous cycle). A single 2500 IU injection of human chorionic gonadotropin (hCG, Chorulon HCG 10 000 IU, Merck Animal Health, USA), which is sufficient to ovulate up to 40–60 follicles in heifers [39], was given coincident with the third PG injection (which is 12 h after the last (or 8th) FSH injection) to induce ovulation (see Figure 1).

The FSH treatment period is hereafter defined as Days 1–4 when heifers received twice-daily FSH injections for 4 days and Day 5 when hCG was injected to stimulate ovulation. Likewise, the post-hCG period encompasses the 9 days post-hCG administration (Days 1–9), during which CL development was monitored.

Blood sampling and ultrasonography

To monitor hormone concentrations, coccygeal vein blood samples (10 mL) were collected twice daily (12 h apart) coincident with FSH injections, then once daily for 2 days beginning at the time of the hCG injection (i.e., 12 h after the last FSH injection), and then once every other day over the course of 9 days. After collection, all blood samples were allowed to clot overnight, and serum was collected and stored frozen at −80 °C until assayed for hormone concentrations. Hormone values for all twice-daily samples were averaged to determine the daily mean. To determine follicle number and size, number of corpora lutea, and uterine thickness, the cattle were subjected daily to serial ovarian and uterine ultrasonography, beginning on the morning of the day of the last PG injection and continuing until the experiment ended, which was 9 days after the hCG injection. Hereafter, follicle sizes are defined as follows: AFC = all follicles ≥3 mm to < 10 mm in diameter and ovulatory-size follicles ≥10 mm in diameter. Uterine thickness was measured from cross sections of both the left and right uterine horns approximately 2 cm from the uterine body bifurcation as previously explained [34, 40].

Immunoassays

Serum concentrations of AMH were determined for blood samples collected daily, beginning on the morning coincident with the first FSH injection and ending the evening of the day for the last FSH injections ($n = 8$ samples per heifer). A commercially available AMH ELISA kit for bovine (MOFA Global, Verona, WI) was used to measure AMH concentrations in duplicate 20 µL serum samples in cattle as per kit instructions. The two-site AMH assay was previously validated [27] for use in cattle and does not cross-react with other members of the transforming growth factor beta (TGFβ) superfamily, including TGFβ, bone morphogenic factor-4 (BMP4), inhibin, or activin [41]. The inter- and intra-assay coefficients of variation for the AMH assay ($n = 6$ plates) were 5.4 and 5.6%, respectively.

Serum estradiol-17β concentrations were determined for blood samples collected daily, beginning on the morning coincident with the first FSH injection and ending on the morning of the hCG injection (12 h after the last FSH injection), and every other day for 9 days, beginning 24 h after hCG ($n = 14$ samples per heifer) by RIA, as previously published [42, 43]. Inter- and intra-assay coefficients of variation for estradiol-17β assays ($n = 15$ plates) were 7.6 and 3.7%, respectively.

Serum concentrations of progesterone were determined for blood samples collected as explained for estradiol ($n = 14$ samples per heifer) as per instructions using a previously validated commercial kit (BC-1113 MP Progesterone Enzyme Immunoassay Test Kit, Catalog, MP Biomedicals Diagnostics Division Orangeburg, NY). The inter- and intra-assay coefficients of variation for progesterone assays ($n = 15$ plates) were 7.8 and 6.8% respectively.

Statistical analysis

All statistical analyses were performed using Statistical Analysis System (SAS 9.4 Institute, Cary, NC) PROC UNIVARIATE and PROC GLIMMIX [44]. The response outcomes analyzed included AFC, ovulatory-size follicle number, CL number, ovulation rate, uterine thickness, and circulating concentrations of AMH, estradiol, and progesterone. In order to render responses that were more normally distributed, AFC, ovulatory-size follicle number, and CL number were analyzed using a square root transformation, whereas uterine thickness, as well as circulating concentrations of AMH, estradiol, and progesterone, was analyzed using a logarithmic transformation. Each response variable was analyzed using a repeated measures mixed model accounting for the main effects of the superovulation

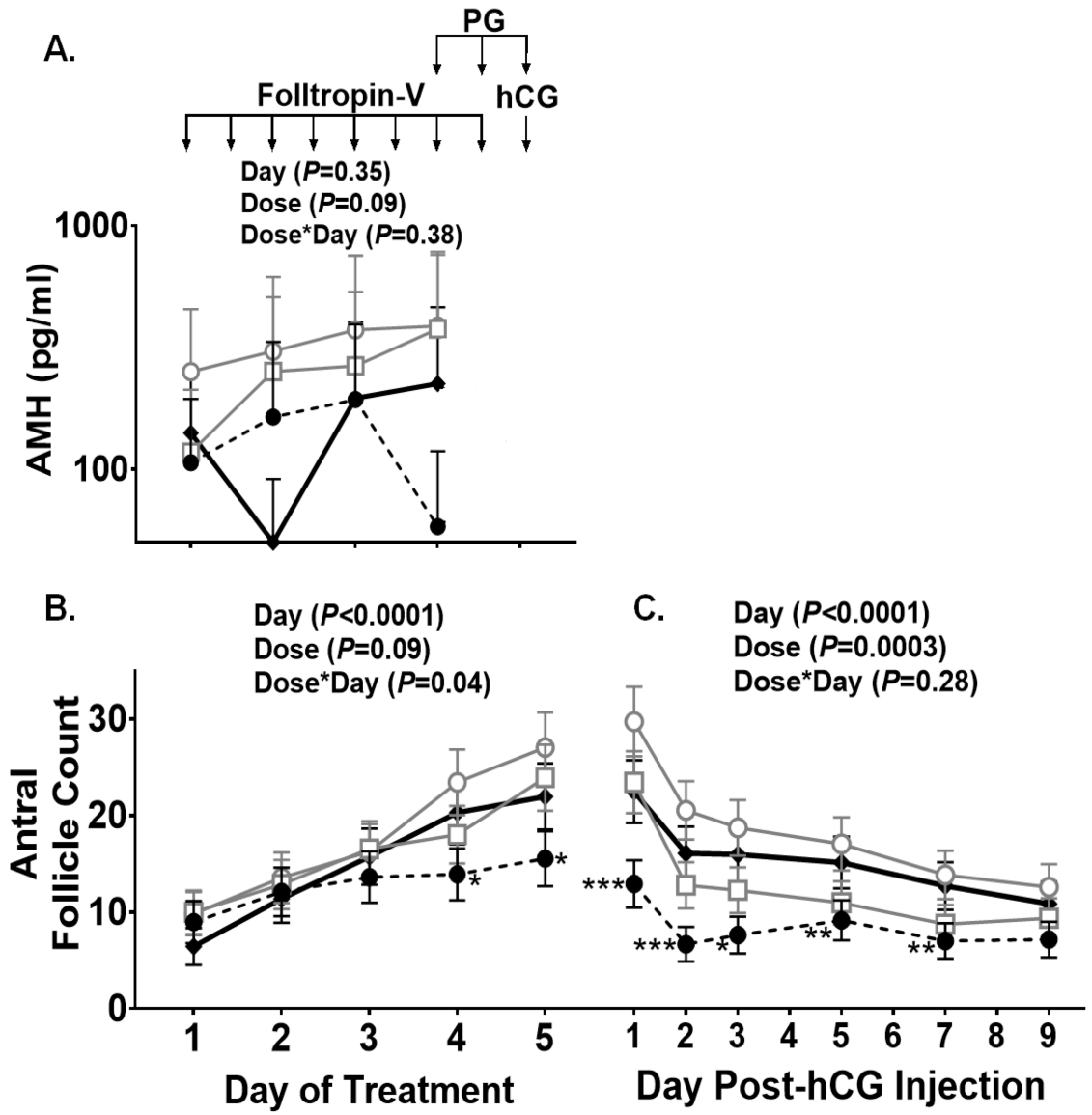

Figure 1. Effect of different doses of FSH on circulating concentrations of AMH and AFC for heifers with a small ovarian reserve. Beginning on Day 1 of the estrous cycle, near the beginning of the first follicular wave, heifers were superovulated with twice-daily injections of each of four different doses (35 IU •, 70 IU □, 140 IU ○, 210 IU ♦) of FSH (Folltropin-V) for 4 days (depicted by arrows), using a Williams Latin Square Design as explained in 'Materials and Methods'. Thus, each heifer was superovulated for a total of four times with 21–24 days between the superovulation regimens. Symbols represent means (±SEM) for the same eight heifers. As depicted by arrows, three prostaglandin $F_{2\alpha}$ (PG) injections were given, 12 h apart, starting on Day 4 of the FSH treatment, to regress the corpus luteum. A single hCG injection (arrow) was given 12 h after the last FSH injection on Day 5 of the treatment to induce ovulation. AMH concentrations were determined every 24 h throughout Folltropin-V treatment but not post-hCG. The number of antral follicles ≥3 to <10 mm in diameter (AFC) was determined daily by ovarian ultrasonography until 3 days after the hCG injection, when AFC was determined every other day until the end of the study. As depicted within each figure, the results of the Type III ANOVA indicate whether significant ($P \leq 0.05$) differences existed in overall AMH concentrations and AFC in heifers during the different days of treatment (Day) and among the FSH doses (Dose), and among heifers treated with the different FSH doses for any day during the FSH treatment period (Day∗Dose). Asterisks indicate that means differ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) when compared with the 210 IU FSH dose.

period, dose, and, where applicable, day of response within the superovulation period and all possible interactions. The repeated measures specification allowed for a stronger correlation among repeated measures across days within superovulation periods, as opposed to across superovulation periods within heifers. The main effects of factors and/or their interactions were considered significant for the corresponding response variable analysis conducted, if $P \leq 0.05$. The results of covariate analysis [44] using all variables measured prior to each superovulation period were not significant. The statistical model also accounted for the first-order carryover effects [45], that is, the effects of the previous superovulation regimen, affecting responses within the current superovulation period on each variable. Any interaction involving the FSH dosage factor that was determined to be statistically significant was analyzed, using the slice statement in SAS [44] to determine mean differences among dosages separately within levels of the other factor involved in the interaction. When the ANOVA test for FSH was significant, Fisher's LSD [44] was used to determine if the highest FSH dose differed significantly ($P \leq 0.05$)

from other FSH doses. All estimated means and standard errors were backtransformed to the scale of observation.

Results

The key strength of the present study's experimental design was that each animal received each of the four FSH doses being tested. We observed no carryover effects ($P > 0.05$) for any response variable, implying no evidence that a superovulation regimen applied within one superovulation period impacted the response within the subsequent superovulation period. In addition, because this study required 8 months to complete, the use of the Williams Latin Square Design minimized potential confounding effects due to aging and weight gain, changes in season and temperature, or other unknown nuisance variables on the response of Holstein heifers to different FSH doses used to induce superovulation. Consequently, study outcomes informed us about the effects of FSH dosage without confounding effects of these other variables.

Effect of FSH dose during superovulation on circulating concentrations of AMH

Circulating concentrations of AMH were measured during Days 1–5 of the FSH treatment. However, AMH concentrations were unaltered during this time period or by the different doses of FSH used to superovulate the heifers (Figure 1A).

Effect of FSH dose during superovulation on AFC

Although AMH concentrations were unaltered during superovulation, AFC increased ($P < 0.0001$) in heifers during Days 1–5 of the FSH treatment (Figure 1B). During Days 1–3 of the FSH treatment, AFC was similar for heifers treated with all different FSH doses. On Days 4 and 5 of the FSH treatment, AFC was similar for heifers treated with 70, 140, and 210 IU FSH doses, but greater ($P \leq 0.05$) for heifers treated with the 210 IU compared with the 35 IU FSH dose (Figure 1B).

During Days 1–9, after hCG administration, AFC decreased ($P < 0.0001$) in heifers treated with the different FSH doses (Figure 1C). Heifers treated with the 70, 140, and 210 IU FSH dose had a similar AFC on Days 1–9 post-hCG. However, heifers treated with the 35 IU FSH dose had a lower ($P \leq 0.05$) AFC on most days post-hCG compared with heifers treated with the 210 IU FSH dose (Figure 1C).

Effect of FSH dose during superovulation on the number of ovulatory-size follicles

The number of ovulatory-size follicles (≥10 mm in diameter) increased ($P < 0.0001$) during Days 1–5 of the FSH treatment for heifers treated with all the different FSH doses (Figure 2A). The number of ovulatory-size follicles was similar for all heifers treated with different FSH doses on Days 1–3 of the FSH treatment. During Days 4 and 5 of the FSH treatment, the heifers treated with the 210 IU FSH dose had a greater ($P \leq 0.05$) number of ovulatory-size follicles compared with heifers treated with the 35 IU FSH dose (Figure 2A). During these same days, however, heifers treated with the 70, 140, and 210 IU FSH doses had a similar number of ovulatory-size follicles (Figure 2A).

In contrast to the increase in number of ovulatory-size follicles during Days 1–5 of FSH treatment, the number of ovulatory-size follicles decreased ($P < 0.0001$) during Days 1–9 post-hCG for heifers treated with all the different FSH doses (Figure 2B). The number of ovulatory-size follicles was similar each day post-hCG for heifers treated with the 70, 140 and 210 IU FSH doses. However, heifers treated with the 210 IU FSH dose had a greater ($P \leq 0.05$) number of ovulatory-size follicles on all days post-hCG compared with heifers treated with the 35 IU FSH dose (Figure 2B).

Effect of FSH dose during superovulation on circulating concentrations of estradiol

Coincident with the increase in AFC (Figure 1B) and number of ovulatory-size follicles (Figure 2A), the circulating estradiol concentrations also increased ($P < 0.0001$) during Days 1–5 of the FSH treatment for heifers treated with all the different FSH doses (Figure 2C). During Days 1–5 of the FSH treatment, estradiol concentrations were similar on Days 1–3 for heifers treated with all the different FSH doses. Estradiol concentrations were also similar on Days 4 and 5 of the FSH treatment for heifers treated with the 35 and 210 IU FSH doses. In contrast, heifers treated with the 210 IU FSH dose had lower ($P \leq 0.05$) estradiol concentrations on Days 4 and 5 of the FSH treatment compared with heifers treated with the 70 IU and 140 IU FSH doses (Figure 2C).

Estradiol concentrations decreased ($P < 0.0001$) in heifers treated with all the different FSH doses after Day 1 post-hCG (Figure 2D). However, heifers treated with the 210 IU FSH dose had higher ($P \leq 0.05$) estradiol concentrations on Day 1 post-hCG compared with the 35 IU FSH dose. In contrast, heifers treated with the 210 FSH dose had lower ($P \leq 0.05$) estradiol concentrations on Day 1 post-hCG compared with the 140 IU FSH dose (Figure 2D).

Effect of FSH dose during superovulation on the number of corpora lutea

The number of detected corpora lutea in heifers by ovarian ultrasonography decreased ($P < 0.02$) for heifers treated with all the different FSH doses during Days 1–5 of the FSH treatment (Figure 3A). However, the number of corpora lutea during this period was unaffected by the different FSH doses (Figure 3A).

During Days 1–9 post-hCG, the number of corpora lutea increased ($P < 0.0001$) for all heifers treated with the different FSH doses (Figure 3D). However, the number of corpora lutea during this period was unaltered by the different FSH doses (Figure 3D).

Effect of FSH dose during superovulation on circulating concentrations of progesterone

Concomitant with the decrease in number of corpora lutea during Days 1–5 of the FSH treatment (Figure 3A), circulating progesterone concentrations also decreased ($P < 0.009$) for heifers treated with the different FSH doses during this same period (Figure 3C). This decrease in progesterone concentrations during Days 1–5 of the FSH treatment was likely in response to the multiple PG injections on Days 4 and 5 of the FSH treatment (Figure 3A). The decrease in progesterone concentrations during Days 1–5 of the FSH treatment, however, was unaltered by the different FSH doses (Figure 3C).

Concomitant with the increase in number of corpora lutea during Days 1–9 post-hCG (Figure 3B), the circulating concentrations of progesterone also increased ($P < 0.0001$) during the same time period. However, the increase in progesterone concentrations post-hCG was unaltered by the different FSH doses (Figure 3D), which is likely explained by the absence of an effect of FSH doses on the number of corpora lutea (Figure 3B).

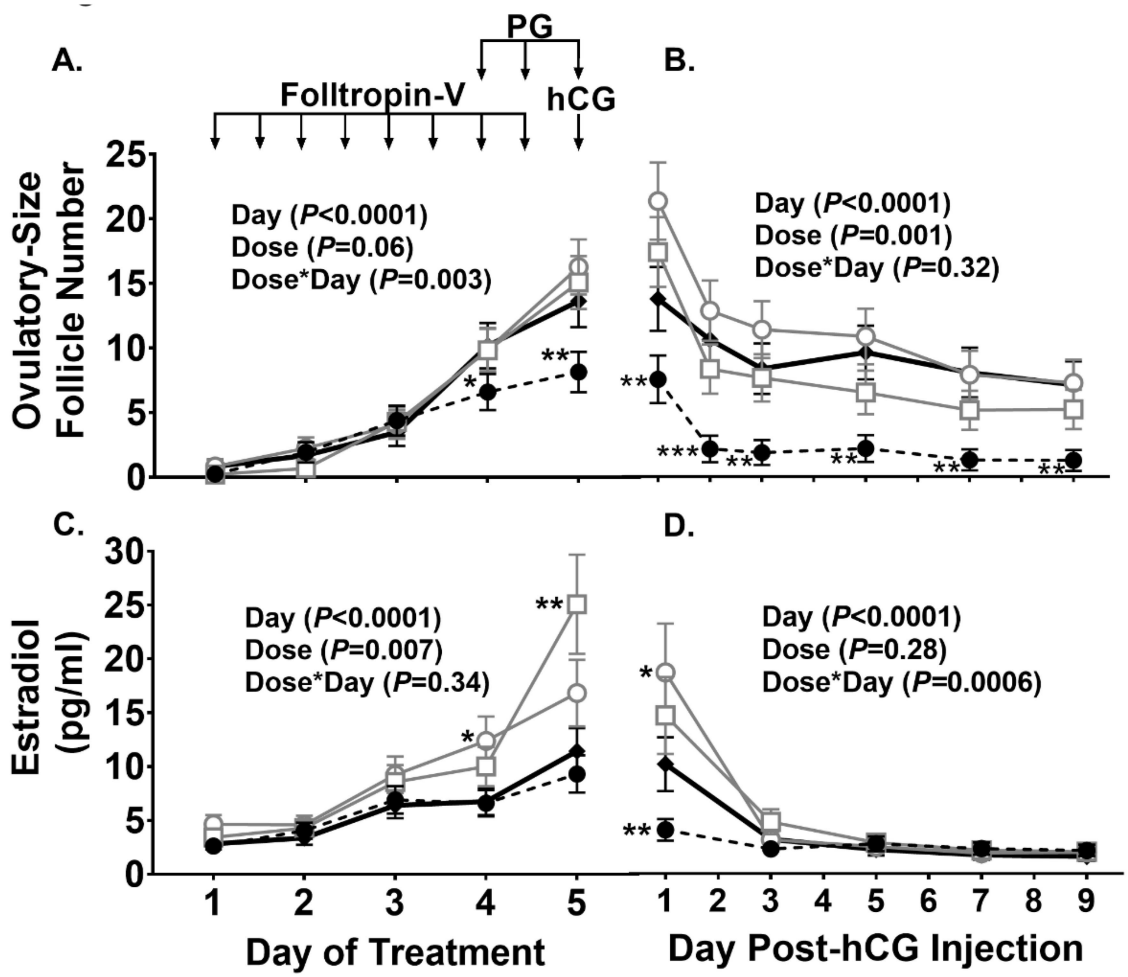

Figure 2. Effect of different doses of FSH on ovulatory-size follicle number and estradiol concentrations for heifers with a small ovarian reserve. The heifers were superovulated, beginning on Day 1 of the estrous cycle with four different doses (35 IU •, 70 IU □, 140 IU ○, 210 IU ♦) of FSH (Folltropin-V, arrows depict injections) and treated with PG and hCG (depicted by arrows) as explained in the legend for Figure 1. Symbols depict means (±SEM) for the same eight heifers. A number of ovulatory-size follicles (≥10 mm) were determined by daily ultrasonography measurements and then every other day, starting 3 days after the hCG injection. Estradiol concentrations were determined at 24-h intervals during FSH treatments and at 48-h intervals post hCG. As depicted within each figure, the results of the Type III ANOVA indicate whether significant ($P \leq 0.05$) differences existed in the overall number of ovulatory-size follicles and circulating estradiol concentrations in heifers during the different days of treatment (Day) and among the FSH doses (Dose), and among heifers treated with the different FSH doses for any day during the treatment period (Day*Dose). Asterisks indicate that means differ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) when compared with the 210 IU FSH dose.

Effect of FSH dose during superovulation on uterine thickness

Uterine thickness was unaltered during Days 1–5 of the FSH treatment or by the different FSH doses used to superovulate heifers (Figure 3E). In contrast, during Days 1–9 post-hCG, uterine thickness decreased ($P < 0.0001$) approximately 35% from its peak thickness on Day 2 (Figure 3F), but the different FSH doses did not alter uterine thickness (Figure 3F).

Effect of FSH dose on ovulation rate

The ovulation rate was calculated by dividing the number of corpora lutea on Day 7 after the hCG injection (Figure 3B) by the number of ovulatory-size follicles (≥10 mm) present at the time of the hCG injection (Figure 2A). Ovulation was not different among the four groups examined individually. However, ovulation rates for heifers treated with the two highest FSH doses combined were lower ($P \leq 0.001$) compared with heifers treated with the two lowest FSH doses combined (Figure 4).

Discussion

This is the first study to address the consequences of different FSH doses on ovarian function within individual animals with comparable ovarian reserves in any mammalian species. The most compelling findings of this study are that higher doses of FSH during superovulation of healthy, nulliparous, young adult Holstein heifers with small ovarian reserves (1) did not result in a dose–response effect for any endpoints of ovulatory follicle function measured, (2) were excessive (lower doses were just as effective) and did not improve follicular growth and response to superovulation, (3) were potentially detrimental to ovulatory follicle function, and (4) did not alter uterine thickness.

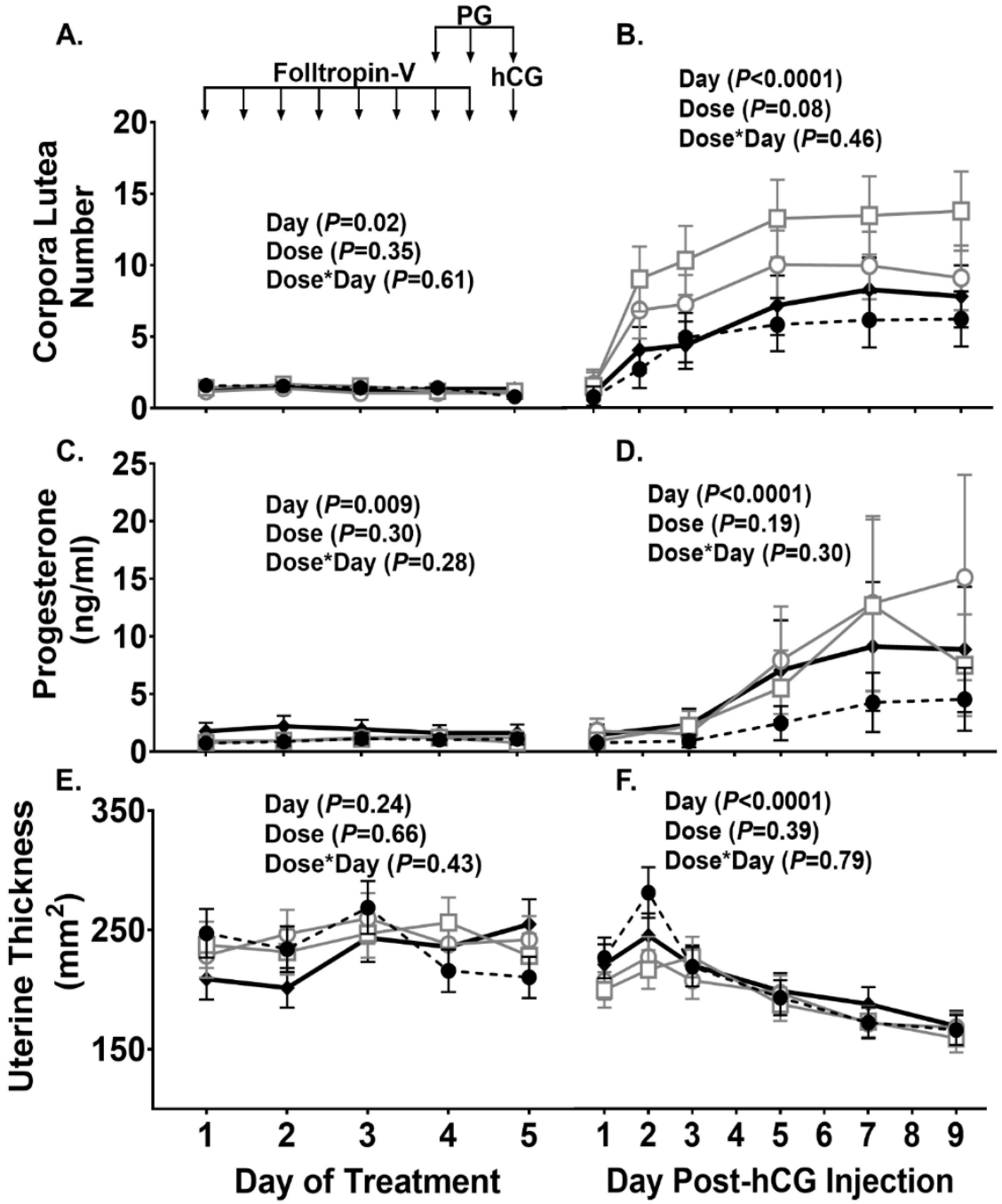

Figure 3. Effect of different doses of FSH on corpora lutea number, progesterone concentrations and uterine thickness for heifers with a small ovarian reserve. Heifers were superovulated, beginning on Day 1 of the estrous cycle with four different doses (35 IU ●, 70 IU □, 140 IU ○, 210 IU ♦) of FSH (Folltropin-V, arrows depict injections) and treated with PG and hCG (depicted by arrows) as explained in the legend for Figure 1. Symbols depict means (±SEM) for the same eight heifers. A number of corpora lutea and uterine thickness were determined by daily ultrasonography measurements and then every other day, starting 3 days after the hCG injection. Progesterone concentrations were determined every 24 h during FSH treatment and every 48 h after the hCG injection. As shown within each figure, the results of the Type III ANOVA indicate whether significant ($P \leq 0.05$) differences existed in the overall number of corpora lutea, progesterone concentrations and uterine thickness for heifers during the different days of treatment (Day) and among the FSH doses (Dose), and among heifers treated with the different FSH doses for any day during the treatment period (Day∗Dose).

FSH has a well-established role in regulating follicular function. FSH action during superovulation is mediated by FSH receptors located exclusively on granulosa and cumulus cells [46]. In addition, the interaction of FSH with its receptors has a critical role in regulation of steroidogenesis, especially estradiol and progesterone production, cumulus cell mass expansion, and cell metabolism, all of which contribute to oocyte competence in ovulatory follicles [46]. Despite the well-established role of FSH in regulating follicular function, the results of the present study did not demonstrate a positive FSH dose–response effect on any of the endpoints measured.

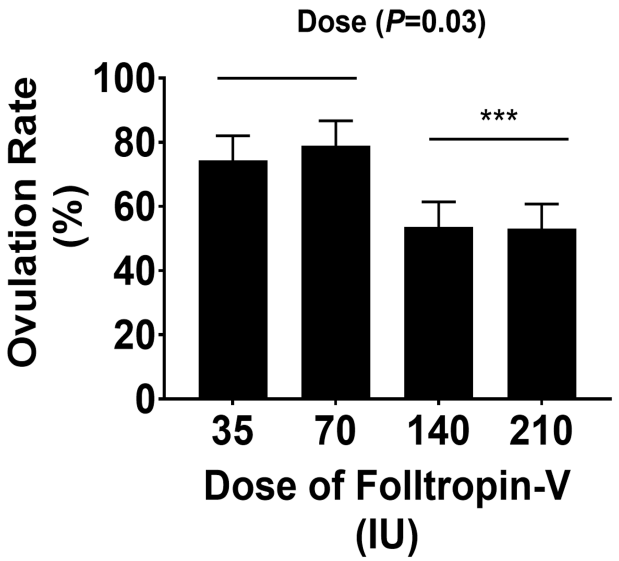

Figure 4. Effect of different doses of FSH on ovulation rate for heifers with a small ovarian reserve. Heifers were superovulated, beginning on Day 1 of the estrous cycle with four different doses (35, 70, 140, 210 IU) of FSH (Folltropin-V, arrows depict injections) and treated with PG and hCG (depicted by arrows) as explained in the legend for Figure 1. Bars depict means (±SEM) for the same eight heifers. Ovulation rate was calculated by dividing the number of corpora lutea on Day 7, post-hCG (Figure 3B) by the number of ovulatory-size follicles present at the time of hCG (Figure 2A). As depicted within the figure, results of the Type III ANOVA indicated that a significant (Dose = $P = 0.03$) difference existed in ovulation rate for heifers treated with the different doses of FSH. Asterisks indicate that the pooled mean (±SEM) for ovulation rates for the 140 and 210 IU doses was lower (***$P < 0.001$) compared with the pooled mean (±SEM) for the 35 and 70 IU doses.

In fact, the highest FSH doses (140 and 210 IU FSH per injection) did not enhance circulating AMH concentrations (marker for growth of healthy preantral and small antral follicles, [10, 47]), AFC (≥3 to <10 mm), numbers of ovulatory-size follicles (≥10 mm), circulating estradiol or progesterone concentrations, number of corpora lutea, or ovulation rate compared with the 70 IU FSH dose. This demonstrates that the highest FSH doses used to superovulate heifers with a low AFC were both excessive and economically wasteful. Interestingly, these high FSH doses did not reduce the number of growing follicles, which may explain why there were no carryover effects, including the impact of the different FSH dose sequences, or of superovulation regimen on the subsequent regimen, for any endpoint measured in the present study.

The reason for the absence of a positive FSH dose–response effect on ovulatory follicle number and function is unknown. However, heifers with a low AFC and small ovarian reserve, as used in the present study, do not respond as well to superovulation as heifers with a higher AFC [24, 26]. These observations in cattle with small ovarian reserves are similar to those observed in women with a small ovarian reserve that also respond poorly to ovarian stimulation protocols during ART [3, 48]. In addition, our previous in vitro FSH dose–response study used granulosa cells isolated from antral follicles [49] that were similar to sizes of the follicles at initiation of superovulation of heifers in the current study. Results of the in vitro study demonstrate that FSH action on granulosa cell function is biphasic, resulting in both a positive and negative window for responsiveness to FSH action. For example, the lower FSH doses showed a positive relationship with estradiol secretion, while the highest doses decreased estradiol secretion. Furthermore, the physiological window of positive responsiveness of granulosa cells to FSH action (i.e., before the onset of premature luteinization and loss of estradiol producing capacity) was positively associated with AFC and size of the ovarian reserve [49]. Thus, the peak response of granulosa cells to FSH action occurs at much lower FSH doses for cattle with a low compared to high AFC [49]. These in vitro observations imply that a much narrower FSH dose range than the one used in the present study will likely be necessary to achieve a positive FSH dose–response for the endpoints measured.

The potential relationship between the AFC and the window for the positive effects of FSH action on granulosa cells during superovulation may also explain the conflicting results among previous FSH dose–response studies [11–14, 28–32] in cattle with an unknown AFC. For example, if the different FSH doses used to superovulate cattle did not span the putative physiological windows for both positive and negative effects of FSH action on granulosa cells [49], the results would be expected to produce either positive, negative, or no effects on endpoints measured.

We also show here that high FSH doses not only fail to improve response to superovulation, but they are also potentially detrimental to ovulatory follicle function. Estradiol is a well-established marker for ovulatory follicle function in cattle [50] and is primarily produced by dominant ovulatory follicles [51, 52]. Thus, the results of the present study imply that the highest dose of FSH used to superovulate heifers impaired ovulatory follicle function. In further support of the potential detrimental impact of the highest FSH dose, the capacity of the low estradiol-producing ovulatory follicles to ovulate in response to an hCG injection was also reduced in the present study. For example, even though the number of ovulatory-size follicles was similar, there was a tendency for the number of CL to be lower for heifers treated with the highest compared with the two intermediate FSH doses. In addition, despite the similar number of ovulatory-size follicles, ovulation rate was lower for heifers treated with the two highest compared with the two lowest FSH doses in the present study. Moreover, the higher number of ovulatory-size follicles detected post-hCG for the heifers treated with the 210 IU FSH dose confirms that these heifers had fewer ovulatory follicles and thus a potentially lower ovulation rate compared with heifers treated with the 35 IU FSH dose. These combined observations illustrate that superovulation of heifers with small ovarian reserves with the highest FSH doses reduced the capacity of ovulatory follicles to produce estradiol, ovulate, and form CL.

This finding is unlikely restricted to the Holstein heifers with a low AFC and small ovarian reserve because other FSH dose–response studies using different breeds and ages of cattle with unknown AFC also show similar results [16, 29]. These observations raise the question of whether the quality of oocytes recovered from ovulatory follicles subjected to high FSH doses is also compromised, which could explain at least part of the high oocyte wastage during ART in cattle [11–17] and women [3, 5, 6, 18–22]. Taken together, these observations indicate that high FSH doses are not only excessive and economically wasteful, but also detrimental to ovulatory follicle function.

Many studies have used doses of Folltropin-V higher than the industry standard of 70 IU per injection to improve the superovulation response or reduce the number of times the cattle are injected to induce superovulation to defray costs associated with embryo transfer [39, 53–55]. However, the two highest FSH doses (140 and 210 IU) per injection in the present study are unlikely to be used commercially to superovulate cattle. In contrast, very

high FSH doses, similar to those in the present study, are likely used during ART cycles in some women. For example, although there was a 6-fold range in total FSH doses used to superovulate heifers (280–1680 IU) in the present study, the total FSH doses during ART cycles in women have a 20-fold range (<1000 to 20 000 IU) [3]. However, the same FSH standard was not used to determine IU for the FSH preparations used in cattle and women. Thus, a direct comparison between the FSH doses used to induce superovulation of heifers in this study and ART cycles in women cannot be established. Nevertheless, relatively high total FSH doses decrease oocyte and embryo quality and yield during embryo transfer in cattle [11, 13, 14], albeit this finding is controversial [28–32]. In addition, high FSH doses are positively correlated with oocyte and embryo wastage in cattle [11–17] and women [3, 5, 6, 18–22] and with the lowest live birth rates during ART cycles in women [3]. Whether decreased ovulatory follicle function following high FSH dose ovarian stimulation protocols, as observed in the present study for the heifers with small ovarian reserves, impairs oocyte quality, remains to be established.

High FSH and LH doses uncouple gonadotropin receptors from their respective signaling system in granulosa, theca, and luteal cells in animal models and humans, altering ovarian function [56–58]. Whether the loss of the capacity of ovulatory follicles to produce estradiol and ovulate is directly attributable to the highest FSH doses used to superovulate heifers could not be unequivocally established here. However, LH stimulates granulosa and luteal cells to produce progesterone [59, 60] and ovulatory follicles to undergo luteinization and produce progesterone during a preovulatory LH surge [59, 60]. Because Folltropin-V is a pituitary source of porcine FSH (pFSH), it contains minor (0.25%) amounts of porcine pituitary LH (pLH). Thus, it is possible that the LH contamination in the highest doses of Folltropin-V caused or contributed to the diminished estradiol-producing capacity of granulosa cells in ovulatory follicles in the present study. Arguing against such an effect, the circulating progesterone concentrations remained low and decreased and were unaltered by different doses of Folltropin-V during the FSH treatment period. Consequently, the minor LH contamination even in the highest dose of Folltropin-V appears insufficient to modify granulosa or luteal cell function. A more plausible explanation for why the highest Folltropin-V doses caused the loss of capacity of ovulatory follicles to produce estradiol and ovulate during superovulation in the present study is that the high Folltropin-V doses triggered premature luteinization and diminished the capacity of granulosa cells in ovulatory follicles to produce estradiol, as observed in our previous in vitro study [49].

Endometrial thickness increases during the follicular phase and declines during the luteal phase of reproductive cycles despite increasing circulating progesterone concentrations in cattle [34] and women [61]. The decrease in endometrial thickness is similar to the patterns shown in the present study for uterine thickness post-hCG. However, the FSH dose and the corresponding changes in ovarian steroid hormone production did not alter uterine thickness. Nevertheless, the pattern of change in uterine thickness in the superovulated low AFC heifers was remarkably similar to changes we previously observed in unstimulated heifers with a high rather than a low AFC [34]. The reason for this difference in patterns among studies is unknown but may be explained by the greater circulating progesterone concentrations in superovulated low AFC heifers in the present study compared with untreated low AFC heifers in our previous study [34]. Although controversial [62, 63], greater endometrial thickness is linked positively to embryo survival in women [64, 65]. Consequently, progesterone therapy is often used during ART in women to enhance luteal progesterone concentrations [66–68] and to improve outcomes, but these results are mixed [69, 70]. The results of the present study in heifers with small ovarian reserves, however, imply that high FSH doses during ART in women are unlikely to diminish endometrial thickness and in turn potentially contribute to poor embryo survival.

The results of the present study combined with the results of others in cattle [11, 12, 14–16, 28–32] and women [3, 5, 6, 18–22] support the conclusion that superovulation of small ovarian reserve cattle with excessive FSH doses is costly and potentially detrimental to ovulatory follicle function. While the detrimental effects of high FSH doses on ovulatory function were established in small ovarian reserve cattle in the present study, it remains to be determined if excessive FSH doses impair oocyte quality and reproductive outcome. Such an effect could help explain poor embryo yield and quality in cattle [11–15] and low live birth rates during ART in women [3].

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Conflict of Interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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